Nuclear magnetic resonance studies of the structure of B50/neuromodulin and its interaction with calmodulin

MINGJIE ZHANG AND HANS J. VOGEL

Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, AB T2N 1N4, Canada

AND

HENK ZWIERS[1]

Department of Medical Physiology/Medical Biochemistry, University of Calgary, Health Sciences Center, 3300 Hospital Drive NW, Calgary, AB T2N 4N1, Canada

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B50/neuromodulin is a neuronal phosphoprotein that is found in association with the inner membrane of nerve cells. In this work, we have studied the structure of bovine B50 in aqueous solution (pH 7.5) by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and our results indicate that B50 is an unstructured protein under these conditions. One-dimensional ^{1}H-NMR titration studies of the interaction between B50 and calmodulin (CaM) have shown that B50 does not interact with (or) interacts very weakly with apo-CaM in solution; neither does B50 interact with Ca^{2+}-CaM. These NMR data are consistent with an earlier observation that B50 is not capable of binding apo-CaM in vitro unless some nonionic detergent is present. We have also detected aromatic NMR peaks for a new posttranslational modification that might involve the His residues of the protein. The interaction of a 14-residue peptide (I38-L51) encompassing the CaM-binding domain of B50 with CaM was also studied by NMR. We have found from two-dimensional transferred nuclear Overhauser enhancement experiments that the B50 peptide binds weakly to apo-CaM in an α-helical conformation; the α-helix appears to be induced by the binding of the peptide to apo-CaM.

Key words: calmodulin, protein B50, neuromodulin, two-dimensional NMR, α-helix.

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La protéine B50 ou neuromoduline est une phosphoprotéine neuronale associée à la membrane interne des cellules nerveuses. Dans le présent travail, nous avons étudié la structure de la protéine B50 de boeuf, en solution dans l'eau (pH 7,5), par spectroscopie de résonance magnétique nucléaire protonique (^{1}H-RMN). Nos résultats démontrent que la protéine B50 est une protéine sans structure ordonnée dans ces conditions. L'étude de l'interaction entre la protéine B50 et la calmoduline (CaM) par titration ^{1}H-RMN unidimensionnelle montre que la protéine B50 ne se lie pas ou ne se lie que très faiblement avec l'apo-CaM en solution, et que la protéine B50 ne se lie pas à la Ca^{2+}-CaM. Ces résultats de RMN sont compatibles avec une observation antérieure, à savoir que la protéine B50 est incapable de se lier à l'apo-CaM in vitro, sauf si un détergent non ionique est présent. Nous avons également détecté des pics de RMN attribuables à des résidus aromatiques, ce qui indique une nouvelle modification posttraductionnelle, probablement sur des résidus His de cette protéine. Nous avons également étudié, grâce à la RMN, l'interaction entre la CaM et un peptide de 14 acides aminés (I38-L51) qui inclut le domaine de liaison de la protéine B50 à la CaM. Au cours d'expériences d'accroissement nucléaire Overhauser transféré en deux dimensions, nous avons observé que le peptide dérivé de B50 se lie faiblement à l'apo-CaM et a une conformation α-hélicoïdale; l'hélice α serait induite par la liaison du peptide à l'apo-CaM.

Mots clés : calmoduline, protéine B50, neuromoduline, RMN bidimensionnelle, hélice α.

[Traduit par la rédaction]

Introduction

B50, also known as neuromodulin, is a 226–239 amino acid neuronal phosphoprotein. It has an apparent molecular mass of 43–67 kDa on SDS–PAGE, but an actual molecular mass of 24–25 kDa as determined by chemical or cDNA sequencing (for reviews, see Liu and Storm 1990; Coggins and Zwiers 1991). Depending on the species, the protein has a slightly different length; the bovine protein that we have studied here contains 239 amino acid residues. Dramatic increases in the concentration of this protein have been consistently associated with neuronal growth and plasticity, as well as successful axonal regeneration (for a review, see Skene 1989). Located almost exclusively to the cytoplasmic face of the neuronal plasma membrane, B50 is a highly acidic (pI 4.5) membrane-associated protein that requires extraction with nonionic detergent (Alexander et al. 1987) or alkali (Coggins et al. 1991) in the initial stages of its purification. The protein contains a single site (Ser41) that can be phosphorylated in vitro by either protein kinase C (Coggins and Zwiers 1989; Apel et al. 1990) or phosphorylase kinase (Paudel et al. 1993). Experiments performed using cultured cells indicate that there may be additional phosphorylation sites in vivo (Spencer et al. 1992). The protein has an unusual amino acid composition; for example, it contains one Phe and three His as the only aromatic amino acids. Hydrodynamic and CD studies of B50 indicate that it has an elongated shape with little secondary structure (Masure et al. 1986).

ABBREVIATIONS: NMR, nuclear magnetic resonance; CaM, calmodulin; kDa, kilodaltons; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; CD, circular dichroism; NOE, nuclear Overhauser enhancement; HPLC, high performance liquid chromatography; dp-B50, dephosphorylated B50; TFE, trifluoroethanol; TRNOE, transferred NOE; NOESY, NOE spectroscopy; TOCSY, total correlated spectroscopy.

[1]Author to whom all correspondence should be addressed.

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Unlike the majority of CaM-binding proteins, B50 has been shown to bind to apo-CaM with a higher affinity than to Ca^{2+}-CaM (Alexander et al. 1987). The CaM-binding domain of B50 has been localized to the N-terminal end of the protein. A peptide encompassing amino acid residues 39–55 was found to bind to CaM with a similar affinity as intact B50 protein in the absence of Ca^{2+} (Alexander et al. 1988). The higher affinity of B50 for apo-CaM and the high cellular concentration of B50 led Liu and Storm (1990) to propose that the function of B50 in the cell is to sequester CaM in the vicinity of CaM-activated enzymes under conditions of low intracellular Ca^{2+} concentration. An increase of the intracellular Ca^{2+} concentration in a stimulated cell would cause the release of CaM from B50. Phosphorylation of Ser41 abolishes the binding of B50 to CaM (Apel et al. 1990), suggesting that the activation of protein kinase C may also lead to the release of CaM from B50. B50 can also interact with acidic phospholipids, and the phospholipid-binding site overlaps with the CaM-binding site on B50 (Houbre et al. 1991), suggesting that the interaction between B50 and CaM mainly relies on electrostatic interactions between the acidic residues on the surface of CaM and the basic residues in the CaM-binding region of B50. The predominance of electrostatic interactions is unusual, since in the majority of cases hydrophobic interactions provide the main driving force for the binding when CaM interacts with target enzymes such as myosin light chain kinases (Ikura et al. 1992; Meador et al. 1992) and nitric oxide synthase (Zhang and Vogel 1994*a*).

In the present work, we have studied the secondary structure of B50 by means of CD and NMR spectroscopy. The possible interaction of B50 with CaM in the absence of KCl has also been investigated by titrating B50 with CaM. Furthermore, the interaction of CaM with a synthetic peptide encompassing the CaM-binding domain of B50 was characterized by CD and NMR, and the CaM-bound structure of the B50 peptide was determined by two-dimensional transferred NOE NMR spectroscopy. This CaM-bound structure was compared with the structure of the peptide in solution.

Materials and methods

Batches (~5 mg) of bovine B50 were purified by alkali extraction and heat treatment of a bovine brain particulate fraction as previously described (Coggins and Zwiers 1989). The crude protein extract was purified by HPLC and the mixture of dp-B50 and dP-50 was further purified by CaM-Sepharose affinity chromatography as previously described (Coggins and Zwiers 1990). After a final round of reverse-phase HPLC, the pure dp-B50 was lyophilized several times in H_2O and stored dry at −80°C prior to use. The protein was >99% pure as judged by the absence of other bands on an overloaded gel stained with silver stain. Moreover, no evidence for any contaminants were found during amino acid sequencing of the protein. The protein concentration of each subsequent batch was measured by a combination of quantitative amino acid analysis and A_{206} of previous quantified B50 standards. Bovine CaM was expressed in and purified from *Escherichia coli* as previously described (Zhang and Vogel 1993). Apo-CaM was obtained by passing a CaM sample through a Chelex-100 column (20 × 1 cm) equilibrated with 100 mM NH_4HCO_3 (pH 8.0).

A 14-residue peptide (NH_2-IQASFRGHITRKKL-COOH) encompassing the CaM-binding domain of B50 was obtained commercially from the peptide synthesis facility at the University of Alberta, Edmonton. The purity of the peptide was greater than 95% as judged by amino acid analysis and HPLC.

CD spectroscopy

CD spectroscopy was performed on a Jasco J-500 spectropolarimeter using a cell path length of 1 mm at 20°C. CD spectra of the B50 peptide with different amounts of TFE were obtained using a 15-μM sample of the peptide in 5 mM citric acid buffer (pH 5.0); and CD spectra of CaM and the CaM–peptide complex (1:1) were measured using 11 μM CaM in 5 mM Tris buffer (pH 7.5) with either 0.5 mM Ca^{2+} or 2 mM EDTA. Two samples of the intact B50 protein in 5 mM Tris buffer (pH 7.5) with and without 0.5% (v/v) Lubrol detergent were also studied by CD spectroscopy.

Sample preparations for NMR experiments

For NMR studies of the peptide in aqueous solution, two samples, one in 90% H_2O – 10% D_2O and the other in 99.9% D_2O, were prepared. The concentration of the peptide was about 4 mM. The pH of the samples was adjusted by the addition of the appropriate amounts of diluted KOD or DCl. All the reported pH values were direct readings from the pH meter. The samples for the TRNOE experiments were prepared by adding aliquots of a 1.5 mM apo-CaM stock solution to the samples described above. The pH titration of B50 was performed in D_2O; about 3 mg of B50 was dissolved in 400 μL of D_2O and the pH of the sample was adjusted by KOD and DCl. No salt was added in the B50 solution.

NMR spectroscopy

All the NMR spectra were acquired on a Bruker AMX500 spectrometer equipped with a 5-mm inverse detection probe head. For the titration of B50 with apo-CaM, 3 mg of B50 was dissolved in 400 μL of D_2O (pH 7.5) and the solution was titrated with a 1.5 mM apo-CaM stock solution at the same pH. No salt was added in either of the samples to promote conditions for detecting the interactions between these two proteins (see below). NOESY (Bodenhausen et al. 1984) and TOCSY (Bax and Davis 1985) spectra were recorded for B50 both in H_2O (pH 7.0) and D_2O (pH 7.5) at 25°C, with mixing times of 300 ms for the NOESY and 60 ms for the TOCSY, respectively. The NMR spectra of the B50 peptide and the CaM–peptide complex were recorded at 5°C. TOCSY (80 ms mixing time) and NOESY (300 ms mixing time) spectra were recorded for the B50 peptide samples both in H_2O and D_2O.

For all of the one-dimensional ^{1}H-NMR experiments, a sweep width of 6000 Hz with 16K data points was used. The two-dimensional ^{1}H-NMR spectra were recorded in the phase-sensitive mode, which was obtained by the time-proportional phase increment technique. Typically, each two-dimensional spectrum contains 512 free induction decays covering a 5500-Hz sweep width with 2K data points in the F2 dimension. For spectra recorded in H_2O, a weak selective presaturation pulse was applied to suppress the H_2O signal. All of the spectra were processed on an X32 computer using the Bruker UXNMR software package. For two-dimensional spectra, one-time zero filling was applied in F1 and a sine-squared window function with a 60° phase shift was applied before Fourier transformation.

Results

Structural characterization of B50

One- and two-dimensional ^{1}H-NMR spectroscopic techniques were used to investigate the structure of B50 in solution. Figure 1 shows a one-dimensional ^{1}H-NMR spectrum of B50 acquired in D_2O (pD 7.5). It is obvious that there is very little chemical shift dispersion in the aliphatic region of the NMR spectrum. A two-dimensional NOESY spectrum of B50 in H_2O showed that no dNN($i,i+1$) NOE cross-peaks could be observed, indicating that B50 does not have any regular α-helical structure. The CD spectrum of B50 in 5 mM Tris buffer gave a typical random coil spectrum for the protein (data not shown). The addition of the detergent

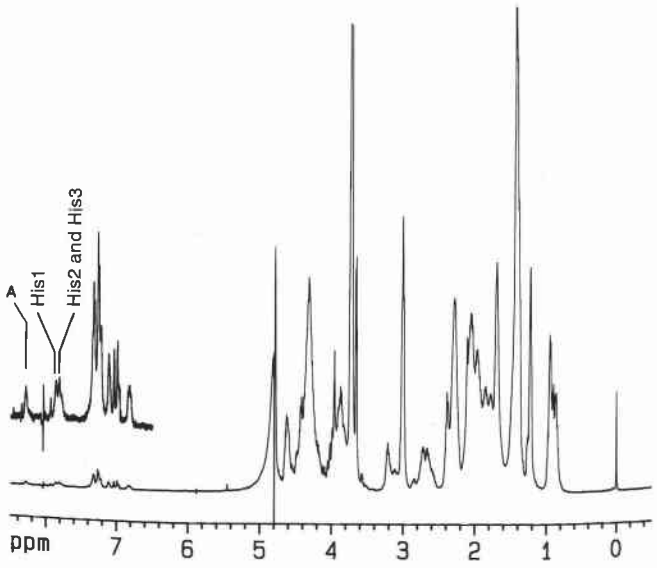

FIG. 1. ^{1}H-NMR spectrum of B50 recorded in D_2O (pD 7.5) at 25°C. A scaled-up plot of the aromatic region is also presented in the figure. The three His residues are labeled as His1, His2, and His3, and the extra pH-dependent resonance is indicated as peak A.

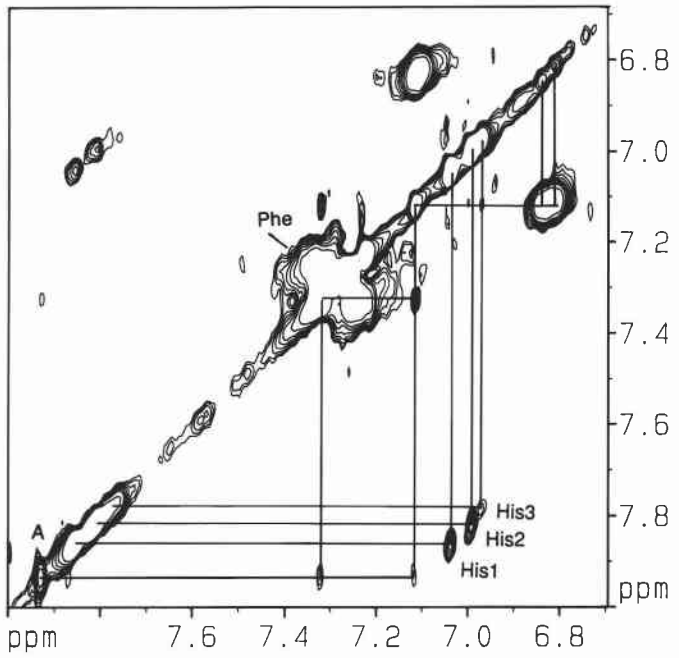

FIG. 2. The aromatic region of the TOCSY spectrum of B50 in D_2O (pD 7.5) with a mixing time of 60 ms. The coupling networks of the three His residues and peak A are indicated in the figure.

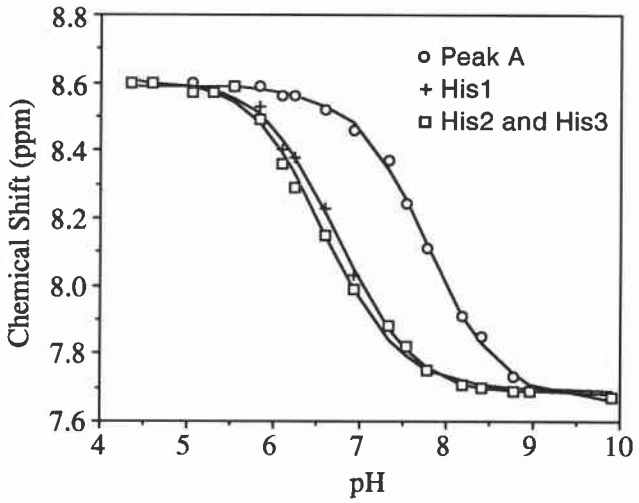

FIG. 3. pH titration curves of the three His residues and peak A in B50. The drawn curves represent the best fit to the data.

Lubrol up to 0.5% (w/v) induced no changes in the CD spectrum (data not shown). All of the results described above suggest that B50 does not have a substantial amount of regular secondary structure in solution. To be specific, we could find no evidence for the presence of any β-sheet structures in the protein that had been suggested by Masure et al. (1986). There were no downfield shifted α-protons in the ^{1}H-NMR spectrum (Fig. 1); this is a very sensitive parameter for the presence of β-sheet structure in proteins (Wishart et al. 1991; Spera and Bax 1991).

Bovine B50 contains three His and only one other aromatic residue, Phe. However, the absorption coefficient at 280 nm for the protein was much higher than could be accounted for by one Phe residue (data not shown; Masure et al. 1986), indicating that the protein contains chromophore(s) other than Phe. Furthermore, the aromatic region of the ^{1}H-NMR spectrum of B50 was more complicated than could be accounted for by just three His and one Phe (Fig. 1). The TOCSY spectrum of B50 in D_2O shows that there were at least five extra resonances in the aromatic region of the spectrum (Fig. 2). Most of these five resonances were coupled with each other, suggesting that the protein may contain heteroaromatic rings that result from posttranslational modification. pH titration of the protein showed that the three His residues had pK_a values of 6.7, 6.6, and 6.6, respectively (Fig. 3), and the resonance at the lowest field (designated peak A in Fig. 1) was also titratable and had a pK_a of 7.7. The pH titration behaviour of peak A was very similar to that of the His residues in the protein, indicating that there may be modified His residues in the protein. Indeed the integration of the His residues in ^{1}H-NMR spectrum (Fig. 1) indicates that the area for the His residues is less than three protons. However, further biochemical characterization is needed to identify the final structure of this posttranslational modification.

Titration of B50 with apo-CaM up to a 1:1 ratio did not induce observable changes in the B50 spectrum and there were no appreciable changes in the apo-CaM spectrum (Fig. 4). It has also been shown by CaM affinity chromatography that B50 will not bind to CaM unless some neutral detergent such as Lubrol is included in the buffer (Coggins and Zwiers 1994). Thus, we concluded that the interaction between B50 and apo-CaM in pure water solution is very weak or not present. As expected, B50 did not interact with Ca^{2+}-CaM, as the ^{1}H-NMR spectrum of B50–Ca^{2+}-CaM (1:1) simply equaled the sum of the ^{1}H-NMR spectra of the two separate proteins (Fig. 4).

Interaction of the B50 peptide with CaM

Since B50 interacts at best only weakly with apo-CaM in pure water solution, it is important to establish whether the reported CaM-binding domain of B50 will bind at all to CaM. Fluorescence spectroscopy has been used to show that

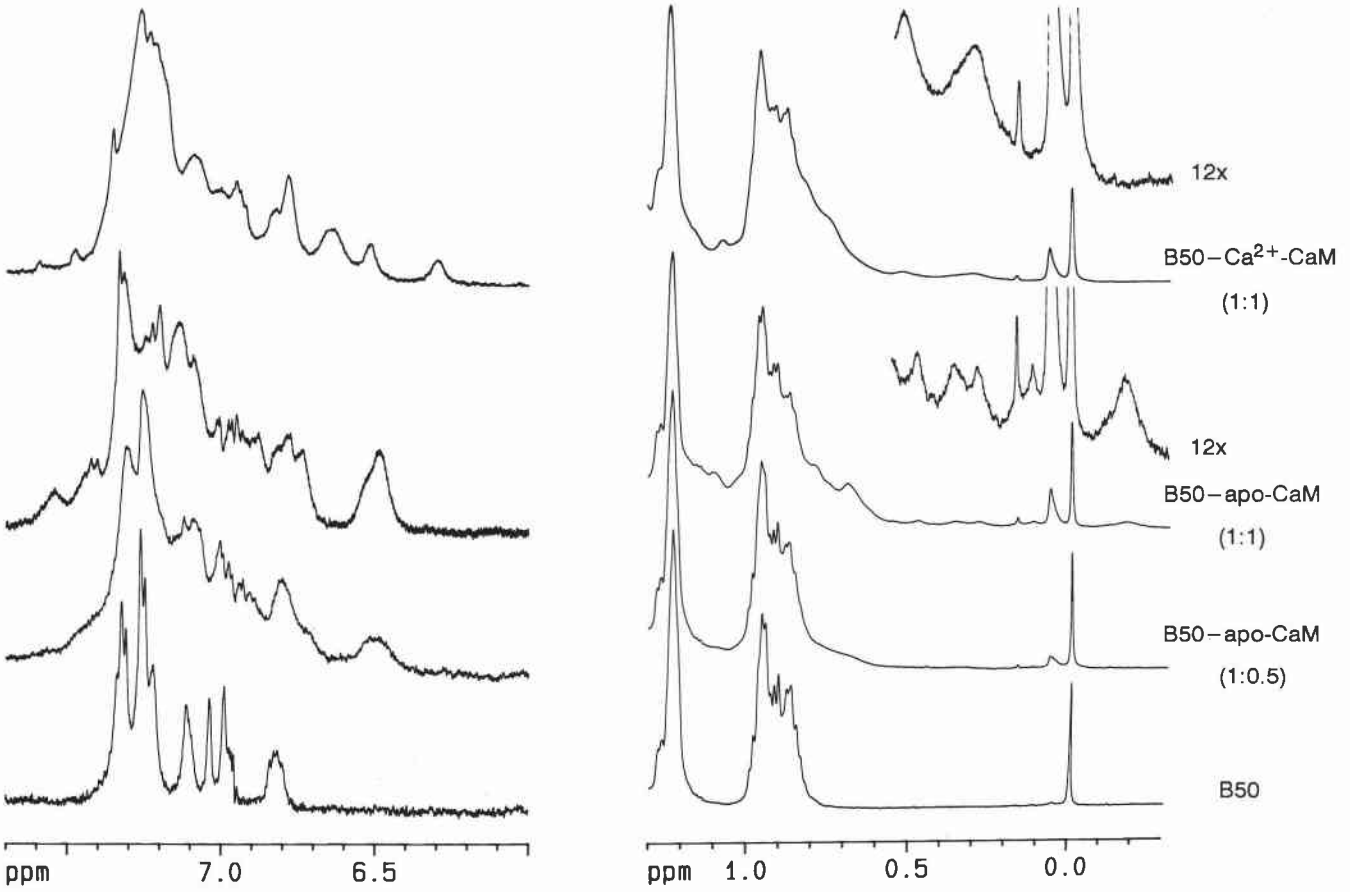

FIG. 4. ^{1}H-NMR titration of B50 with apo-CaM and Ca^{2+}-CaM. Only the aromatic region and the upfield shifted methyl region of the spectra are shown here.

a peptide from B50 (Q39-K55) with a Trp substitution for Phe42 can bind to apo-CaM with an affinity of 0.41 μM. Competition experiments of this Trp-substituted peptide with the native peptide showed that the native peptide had a weaker affinity of ≈4.2 μM for apo-CaM (Alexander et al. 1988). In this study, we used a similar native peptide (I38-L51) to study the interaction with CaM.

The TRNOE method is very useful for determining the structure of small, protein-bound peptides (Campbell and Sykes 1993). Figure 5 shows that the B50 peptide indeed binds to apo-CaM. The B50 peptide alone in solution had very weak NOE cross-peaks because of its short correlation time (τ_c), and at this specific pH (pH 6.4) no NH/NH NOE cross-peaks could be observed (Fig. 5A). The addition of apo-CaM at a 1:25 molar ratio induced a significant change in the NOESY spectrum of the peptide. The intensity of the NH/side-chain NOE cross-peaks increased significantly and several dNN($i,i+1$) NOE cross-peaks were also observed (Fig. 5B). The stronger NOE cross-peaks in Fig. 5B resulted from the magnetization transfer from the CaM-bound peptide to the free peptide in the solution, since the CaM-bound peptide had a much longer τ_c compared with the free peptide. These results shown in Fig. 5 clearly indicate that the CaM-bound and free B50 peptides are exchanging at a fast to intermediate rate. This binding is specific for apo-CaM, since addition of Ca^{2+}-CaM to the B50 peptide solution had no effect on the NOESY spectrum of the peptide (data not shown).

It is possible to determine the CaM-bound structure of the B50 peptide by the transferred NOE technique based on Fig. 5. We performed the experiments at two different pH values (6.4 and 6.0) to partially resolve some resonance overlap in the amide region. The TOCSY spectrum of the peptide in H_2O was used to assign the spin systems of the amino acid residues and NOESY experiments in H_2O served to trace out the sequential connectivities of the peptide. Figure 6A shows the fingerprint region of the B50 peptide in the presence of 1:25 molar ratio of apo-CaM at pH 6.0 and the sequential assignment is indicated for the individual amino acid residues. Table 1 gives a list of the chemical shifts of the peptide at pH 6.0 as derived from the TRNOE experiment. Figure 6B presents the amide region of the NOESY spectrum at pH 6.0; several sequential amide NOE cross-peaks could be observed. In addition to the NOE peaks in Fig. 6B, we observed dNN($i,i+1$) NOE cross-peaks from Ser4 to Phe5 and Phe5 to Arg6 for the same peptide–CaM mixture at pH 6.4. TRNOE experiments on the B50 peptide and apo-CaM mixture in D_2O were used to find medium range NOE connectivities, and several dαβ($i,i+3$) NOE cross-peaks were detected. Figure 7 gives the summary of the NOE pattern for the B50 peptide that was derived from the TRNOE experiments; the observed NOE pattern suggests that the B50 peptide binds to apo-CaM with an α-helical conformation. The α-helix of the CaM-bound B50 peptide runs from Ala3 to Leu14. The CD spectrum of the apo-CaM–peptide (1:1) complex shows very subtle differences from that of apo-CaM (data not shown). This has also been seen for the CaM-binding peptide from caldesmon, which also binds relatively weakly to CaM (Zhang and Vogel 1994*b*).

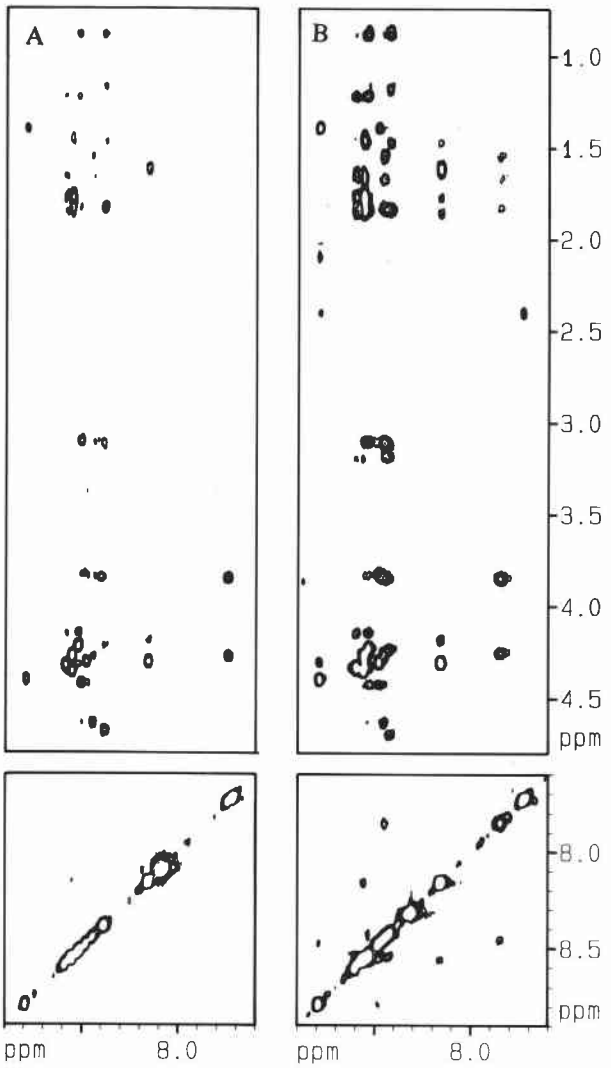

FIG. 5. The NOESY spectra (300 ms mixing time) of the B50 peptide in H_2O acquired in the absence (A) and presence (B) of a small amount of (1:25 molar ratio) apo-CaM. The spectra were recorded at pH 6.4 and 25°C.

The structure of the B50 peptide in water

Peptides encompassing the CaM-binding domains of some enzymes such as myosin light chain kinase are unstructured in aqueous solution, but an α-helix is induced by the binding of the peptide to Ca^{2+}-CaM (Ikura et al. 1992; Zhang et al. 1993). For some other CaM-binding domains, such as the synthetic peptide from the constitutive nitric oxide synthase, the peptide adopts a nascent α-helical structure in solution and binds to CaM in the same α-helical conformation, which is more stable (Zhang and Vogel 1994*a*). Here we also studied the conformation of the free B50 peptide in solution by NMR. As we pointed out in Fig. 5A, no dNN($i,i+1$) NOE cross-peaks of the free peptide were seen in H_2O at pH 6.4. However, when the pH of the sample was lowered to 5.0 and a long mixing time (e.g., 300 ms) was used in the NOESY experiment, we observed some weak NOE cross-peaks in the amide region (Fig. 8). The pattern of the dNN($i,i+1$) NOE for the free peptide was the same as that for the peptide derived from TRNOE experiments. However, no medium range dαβ($i,i+3$) NOEs, which are diagnostic for α-helical turns, were found for the peptide in D_2O solution

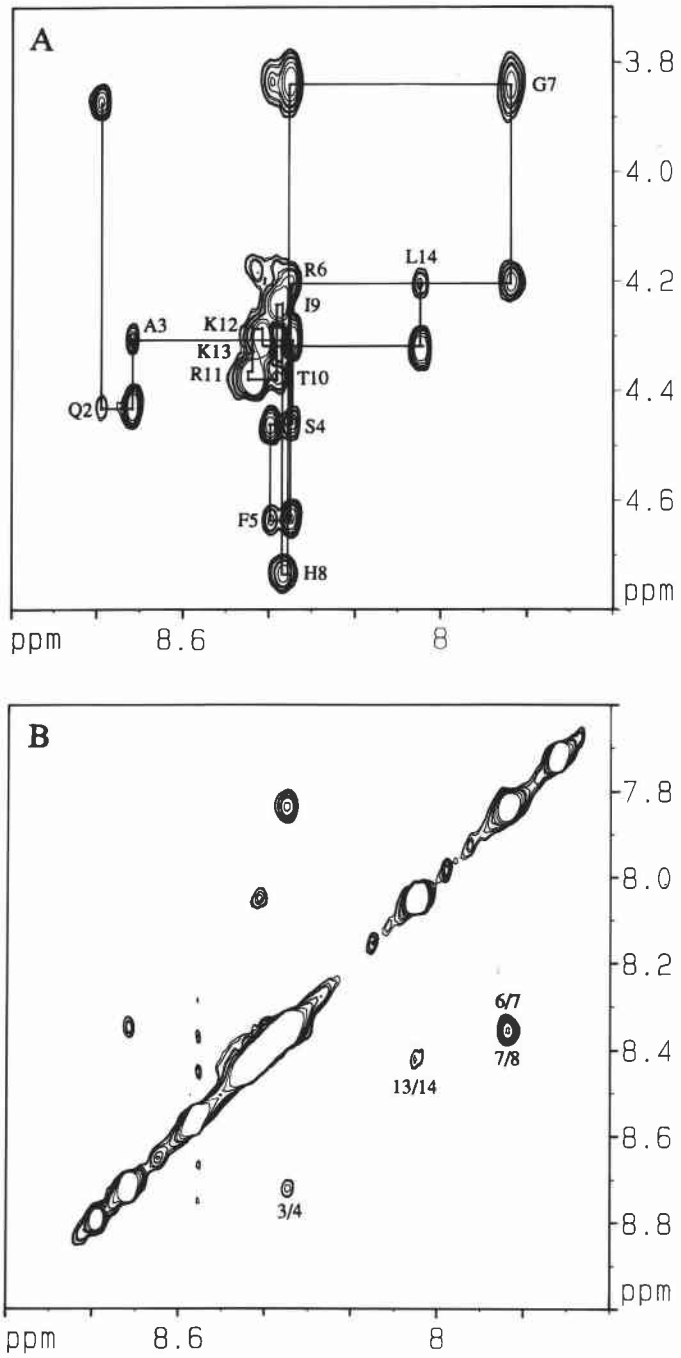

FIG. 6. (A) Fingerprint region of the NOESY spectrum of the B50 peptide from the TRNOE experiment showing the sequential assignment of the peptide; (B) the amide region of the same NOESY spectrum.

under these conditions. The above results suggest that the peptide adopts a random coil structure in water. The addition of the α-helix promoting solvent TFE up to 25% (v/v) did not provide any additional NOE connectivities (data not shown), reinforcing the notion that the peptide in solution adopts a random coil structure. CD spectra also indicated that there is no detectable α-helical structure for the peptide in water solution, which is common for a short linear peptide (data not shown). The addition of TFE up to 50% also did not induce appreciable changes in the CD spectra of the peptide (data not shown).

TABLE 1. ^{1}H-NMR chemical shift values of the B50 peptide at pH 6.0 derived from TRNOE experiment

	NH	αH	βH	Other
I1	nd[a]	3.87	1.97	γCH_2:1.25,1.52 γCH_3:0.99 δCH_3:0.92
Q2	8.88	4.39	2.09,2.02	γCH_2:2.40 δNH_2:7.73,7.03
A3	8.79	4.30	1.39	
S4	8.48	4.42	3.82	
F5	8.55	4.63	3.14,3.14	2,6H:7.26 3,5H:7.32 4H:7.28
R6	8.45	4.25	1.83,1.67	γCH_2:1.55,1.55 δCH_2:3.20,3.20
G7	7.85	3.85		
H8	8.45	4.70	3.18,3.10	2H:8.55 4H:7.25 δCH_2:3.17
I9	8.42	4.23	1.83	γCH_2:1.46,1.17 γCH_3:0.87 δCH_3:0.87
T10	8.54	4.32	4.14	γCH_3:1.21
R11	8.60	4.33	1.85,1.76	γCH_2:1.64,1.64 δCH_2:3.20,3.20
K12	8.56	4.35	1.85,1.76	γCH_2:1.44,1.44 δCH_2:1.69,1.69 ωCH_2:3.00
K13	8.56	4.26	1.85,1.76	γCH_2:1.46,1.46 δCH_2:1.72,1.72 ωCH_2:3.02
L14	8.16	4.18	1.65,1.65	γCH_2:1.64,1.64 δCH_2:0.88,0.88

NOTE: Chemical shifts are expressed in ppm relative to (trimethylsilyl)propionic-d_4 acid (TSP) at 0 ppm.
[a]Assignment not obtained.

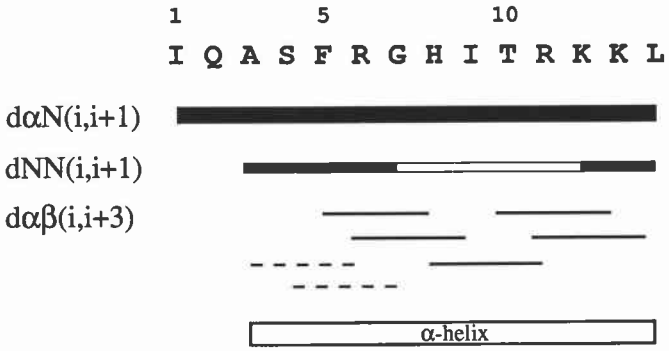

FIG. 7. Summary of the NOE connectivities observed for the B50 peptide from the TRNOE experiment. The open box and dashed lines represent the NOEs that are presumably present but could not be observed owing to spectral overlap.

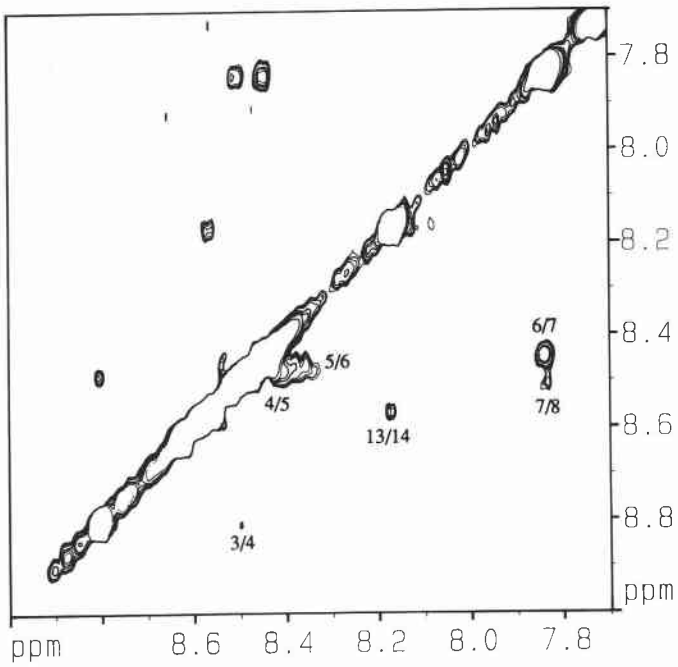

FIG. 8. Amide region of the NOESY spectrum of the B50 peptide in H_2O (pH 5.0) recorded with a mixing time of 300 ms. The cross-peaks are labeled by their amino acid number.

Discussion

In this work, we characterized the structure of B50 and studied the interaction of the protein with CaM in aqueous solution. In agreement with a previous study (Masure et al. 1986), B50 appears to be a largely unstructured protein without any significant amount of regular secondary structure, as indicated by NMR and CD spectroscopy. However, this in vitro situation does not have to be directly related to the situation in vivo, since B50 is a membrane-associated protein and the lipid-rich environment may induce a well-defined structure that is quite different from the one seen in vitro. When the interaction of B50 with CaM was studied by NMR spectroscopy, our data showed that B50 interacts only weakly with apo-CaM in pure water, since titration of B50 with apo-CaM did not induce observable spectral changes for both proteins. This result is consistent with the observation that B50 will only bind to a CaM-Sepharose column, provided that a neutral detergent such as Lubrol as well as EDTA are included in the buffer (Coggins and Zwiers 1994). The localization of B50 at the cytosolic side of neuronal membranes (i.e., a lipid-rich environment) would be in line with our in vitro observations, indicating that the interaction of B50 and CaM only occurs under special conditions such as the presence of lipids. However, our data do not exclude the possibility that the interaction between B50 and apo-CaM induces very subtle structural changes on both proteins, but these do not give rise to appreciable spectral changes. The latter possibility seems unlikely, since CaM can induce the formation of an α-helical structure for the CaM-binding domain peptide of B50 (Fig. 5). As expected, no evidence for interactions between intact B50 and Ca^{2+}-CaM was obtained (Fig. 4).

The additional resonances that were observed in the aromatic region of the ^{1}H-NMR spectrum of B50 (Fig. 2) are likely due to a posttranslational modification of the protein. It is highly unlikely that these resonances represent impurities carried over from the purification, since these resonances survive through several cycles of HPLC purification of the protein, and all the chemicals used during purification were checked by NMR and proven to have no relation to these peaks. To date, three posttranslational modifications, i.e.,

palmitylation (Skene and Virag 1989; Chapman et al. 1992), ADP-ribosylation (Coggins et al. 1993), and phosphorylation (Coggins and Zwiers 1989), were identified in B50. It is obvious that phosphorylation would not introduce any additional protons into the protein. ADP-ribosylation will introduce two additional protons in the aromatic region of the protein spectrum recorded in D_2O; however, the chemical shifts, coupling patterns, and the number of protons of ADP-ribose (McDonald et al. 1992) clearly indicate that these additional resonances in B50 do not result from ADP-ribosylation. We have also compared the ^{1}H-NMR spectra of B50 with palmitate and other fatty acids; the results have shown that the additional resonances in B50 do not originate from such compounds (spectra not shown). Thus, we conclude that the additional resonances that we have observed in B50 have no correlation with the known posttranslational modifications identified earlier.

The titration behavior of peak A is extremely similar to that of the His residues in the protein, except that it has a higher pK_a (Fig. 3) which can be the result from a modification of His residues. Peak A also correlates with a peak that is close to the H4's of the other His residues in the TOCSY spectrum (Fig. 2), although the coupling pattern of peak A is clearly more complicated. Most importantly, for a freshly dissolved B50 sample in D_2O, the area of the H2 protons from the His residues is smaller than three protons, while the area summation of peak A and the H2 protons from His roughly equals three protons. Based on the above data, we believe that peak A and its coupling partners result from posttranslationally modified His residues. If these resonances indeed represent modified His residues, then different levels of the modification have occurred in the protein since His3 has the lowest peak intensity among the three His residues. We have observed that B60, which is a fragment of B50 from residue 41 to 239 (Coggins and Zwiers 1990), also has a peak similar to A, indicating that this modification is not specific for the His32 residue in the N-terminal part of B50. It seems that this modified His is heat and acid labile, since it does not survive through amino acid analysis reactions. However, we have not been able to match any currently known posttranslational modified His residues (Graves et al. 1994) with the one observed in this work. Hence, we may be dealing with a new posttranslational modification reaction of His residues. Further characterization is needed to identify these modified His residues.

The interaction of the CaM-binding domain of B50 with CaM was investigated by two-dimensional ^{1}H-NMR spectroscopy. In agreement with a previous study (Alexander et al. 1988), the B50 peptide showed very little interaction with Ca^{2+}-CaM, and the interaction between the B50 peptide and apo-CaM was much weaker compared with the CaM-binding domains from other CaM-dependent enzymes such as myosin light chain kinase; the latter binds with a K_d in the nanomolar range. The fast-to-intermediate exchange between the CaM-bound and free B50 peptide in solution allowed us to determine the CaM-bound structure of the B50 peptide by the two-dimensional TRNOE NMR technique. The results showed that the B50 peptide binds to apo-CaM in an α-helical conformation. This α-helical structure is induced by the binding of apo-CaM, since the B50 peptide alone has a random coil structure in H_2O. Although the B50 peptide binds to CaM with an α-helical structure like most other CaM-binding peptides (O'Neil and Degrado 1990; Ikura et al. 1992; Zhang and Vogel 1994*a*, 1994*b*), a helical wheel analysis of the B50 peptide (Chapman et al. 1991) has shown that this helix does not have the amphiphilic property which is characteristic of most CaM-binding peptides (O'Neil and DeGrado 1990). Thus it seems that the B50 peptide interacts with apo-CaM in a distinct manner compared with other Ca^{2+}-CaM – target enzyme complexes. Previous studies of the interaction of B50 or its CaM-binding domain with CaM have shown that acidic phospholipids bind to the CaM-binding region on B50 and that the binding of phospholipids prevents CaM from binding to B50 (Houbre et al. 1991). Furthermore, the introduction of a negative charge(s) at position 41 either by phosphorylation (Alexander et al. 1987; Apel et al. 1990) or by site-directed mutagenesis (Chapman et al. 1991) also results in the dissociation of B50 from apo-CaM; likewise, the affinity of the B50 peptide for CaM can be significantly reduced by increasing the ionic strength (Chapman et al. 1991). These data strongly suggest that electrostatic interactions, which occur between the positive charge clusters on the B50 peptide and the negative charges on apo-CaM, contributes the main binding energy; the hydrophobic interactions of Phe42 with residue(s) from CaM can also be important (Chapman et al. 1991). The predominance of electrostatic interactions could be the reason that B50 only binds to apo-CaM, since the binding of Ca^{2+} to CaM may induce the redistribution of the negative charges on CaM, as well as the neutralization of negative charges by four double positively charged Ca^{2+} ions.

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Alexander, K.A., Cimler, B.M., Meier, K.E., and Storm, D.R. 1987. Regulation of calmodulin binding to P-57. J. Biol. Chem. **262**: 6108–6113.

Alexander, K.A., Wakim, B.T., Doyl, G.S., Walsh, K.A., and Storm, D.R. 1988. Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. J. Biol. Chem. **263**: 7544–7549.

Apel, E.D., Byford, M.F., Au, D., Walsh, K.A., and Storm, D.R. 1990. Identification of the protein kinase C phosphorylation site in neuromodulin. Biochemistry, **29**: 2330–2335.

Bax, A., and Davis, D.G. 1985. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. **65**: 355–360.

Bodenhausen, G., Kogler, H., and Ernst, R.R. 1984. Selection of coherance-transfer pathways in NMR pulse experiment. J. Magn. Reson. **58**: 370–388.

Campbell, A.P., and Sykes, B.D. 1993. The two-dimensional transferred nuclear Overhauser effect: theory and practice. Annu. Rev. Biophys. Biomol. Struct. **22**: 99–122.

Chapman, E.R., Au, D., Alexander, K.A., Nicolson, T.A., and Storm, D.R. 1991. Characterization of the calmodulin binding domain of neuromodulin. J. Biol. Chem. **266**: 207–213.

Chapman, E.R., Estep, R.P., and Storm, D.R. 1992. Palmitylation of neuromodulin (GAP-43) is not required for phosphorylation by protein kinase C. J. Biol. Chem. **267**: 25 233 – 25 238.

Coggins, P.J., and Zwiers, H. 1989. Evidence for a single kinase C-mediated phosphorylation site in rat brain protein B-50. J. Neurochem. **53**: 1895–1901.

Coggins, P.J., and Zwiers, H. 1990. Binding of the neuronal protein B-50, but not the metabolite B-60, to calmodulin. J. Neurochem. **54**: 274–277.

Coggins, P.J., and Zwiers, H. 1991. B-50 (GAP-43): biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. J. Neurochem. **56**: 1095–1105.

Coggins, P.J., and Zwiers, H. 1994. Detergents and peptides alter proteolysis and calmodulin binding of B50/GAP-43 in vitro. J. Neurochem. In press.

Coggins, P.J., Stanisz, J., Nagy, A., and Zwiers, H. 1991. Identification of a calmodulin-binding, B50-immunoreactive C-kinase substrate (BICKS) in bovine brain. Neurosci. Res. Commun. **8**: 49–56.

Coggins, P.J., McLean, K., Nagy, A., and Zwiers, H. 1993. ADP-ribosylation of the neuronal phosphoprotein B-50/GAP-43. J. Neurochem **60**: 368–371.

Graves, D., Martin, B., and Wang, J.H. 1994. Co- and post-translational modification of proteins: chemical principles and biological effects. Oxford University Press, Oxford.

Houbre, D., Duportail, G., Deloulme, J.-C., and Baudier, J. 1991. The interaction of the brain-specific calmodulin-binding protein kinase C substrate, neuromodulin. J. Biol. Chem. **266**: 7121–7131.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B., and Bax, A. 1992. Solution structure of a calmodulin-target peptide by multidimensional NMR. Science (Washington, D.C.), **256**: 632–638.

Liu, Y.L., and Storm, D.R. 1990. Regulation of free calmodulin level by neuromodulin: neuron growth and regeneration. Trends Pharmacol. Sci. **11**: 107–111.

Masure, H.R., Alexander, K.A., Wakin, B.T., and Storm, D.R. 1986. Physicochemical and hydrodynamic characterization of P-57, a neurospecific calmodulin binding protein. Biochemistry, **25**: 7553–7560.

McDonald, L.J., Wainschel, L.A., Oppenheimer, N.J., and Moss, J. 1992. Amino acid-specific ADP-ribosylation: structural characterization and chemical differentiation of ADP-ribose-cysteine adducts formed nonenzymatically and in a pertussis toxin-catalyzed reaction. Biochemistry, **31**: 11 881 – 11 887.

Meador, W.E., Means, A.R., and Quiocho, F. 1992. Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin–peptide complex. Science (Washington, D.C.), **257**: 1251–1254.

O'Neil, K.T., and DeGrado, W. 1990. How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices. Trends Biochem. Sci. **15**: 59–64.

Paudel, H.K., Zwiers, H., and Wang, J.H. 1993. Phosphorylase kinase phosphorylates the calmodulin-binding regulatory regions of neuronal tissue-specific proteins B-50 (GAP-43) and neurogranin. J. Biol. Chem. **268**: 6207–6213.

Skene, J.H.P. 1989. Axonal growth-associated proteins. Annu. Rev. Neurosci. **12**: 127–156.

Skene, J.H.P., and Virag, I. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. J. Cell Biol. **108**: 613–624.

Spencer, S.A., Schuh, S.M., Liu, W.-S., and Willard, M.B. 1992. GAP-43, a protein associated with axon growth, is phosphorylated at three sites in cultured neurons and rat brain. J. Biol. Chem. **267**: 9059–9064.

Spera, S., and Bax, A. 1991. Empirical correlation between protein backbone conformation and C_α and C_β ^{13}C nuclear magnetic resonance chemical shifts. J. Am. Chem. Soc. **113**: 5490–5492.

Wishart, D.S., Sykes, B.D., and Richards, F.M. 1991. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. J. Mol. Biol. **222**: 311–333.

Zhang, M., and Vogel, H.J. 1993. Determination of the side chain pK_a values of the lysine residues in calmodulin. J. Biol. Chem. **268**: 22 420 – 22 428.

Zhang, M., and Vogel, H.J. 1994*a*. Characterization of the calmodulin-binding domain of rat cerebellar nitric oxide synthase. J. Biol. Chem. **269**: 981–985.

Zhang, M., and Vogel, H.J. 1994*b*. The calmodulin-binding domain of caldesmon binds to calmodulin in an α-helical conformation. Biochemistry, **33**: 1163–1171.

Zhang, M., Yuan, T., and Vogel, H.J. 1993. A peptide analog of the calmodulin-binding domain of myosin light chain kinase adopts an α-helical structure in aqueous trifluoethanol. Protein Sci. **2**: 1931–1937.